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EXPRESS MAIL NO.
EL 769971565 US

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>SAK amino acid seq. (SEQ ID NO:2)

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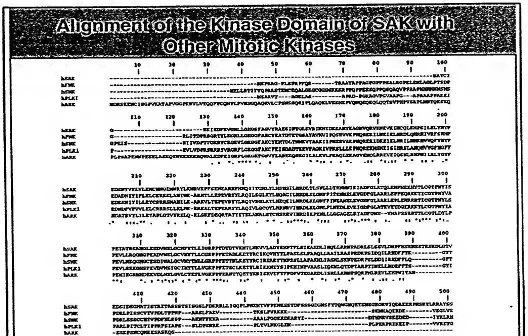
Figure 2



Two hSAK Mutants Generated for the Dominant negative Studies: D154A and K41M

Figure 3

Summary of Target Validation Studies: SAK

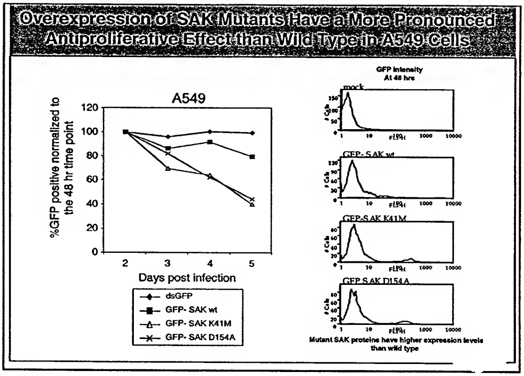
Dominant negative studies

Antiproliferative Activity	**Tumor** A549	Hela	PC-3	MCF7	H1299	**Normal** HMEC	PrEC
Wt							
GFP fusion	+	+	++	+/-	+/-	+/-	+/-
IRES GFP	+	+		+/-	nd	+/-	nd
K41M							
GFP fusion	++	++	++	+	+/-	+/-	+/-
IRES GFP	++	++	++	+	nd	+/-	nd
D154A							
GFP fusion	++	nd	++	+	+/-	+/-	+/-
IRES GFP	++	nd	++	+	nd	+/-	nd

Antisense:	Hela	A549	H1299
	+	+/-	+/-

(+ indicates antiproliferative effect in either the GFP positivity study, cell tracker or antisense studies)

Figure 4



Overexpression of SAK Mutants Have a More Pronounced Antiproliferative Effect than Wild Type in A549 Cells
A549
%GFP positive normalized to the 48 hr time point
120
100
80
60
40
20
0
2
3
4
5
Days post infection
dsGFP
GFP- SAK wt
GFP- SAK K41M
GFP- SAK D154A
GFP intensity
At 48 hrs
mock
GFP- SAK wt
GFP-SAK K41M
GFP SAK D154A
1
10
100
1000
10000
Mutant SAK proteins have higher expression levels than wild type

Figure 5

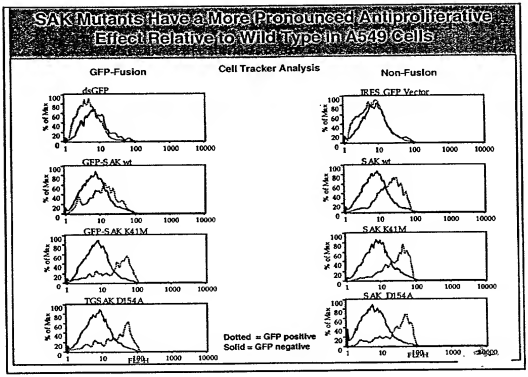

SAK Mutants Have a More Pronounced Antiproliferative Effect Relative to Wild Type in A549 Cells
GFP-Fusion
Cell Tracker Analysis
Non-Fusion
dsGFP
GFP-SAK wt
GFP-SAK K41M
TGSAK D154A
IRES GFP Vector
SAK wt
SAK K41M
SAK D154A
% of Max
FL2-H
Dotted = GFP positive
Solid = GFP negative

Figure 6

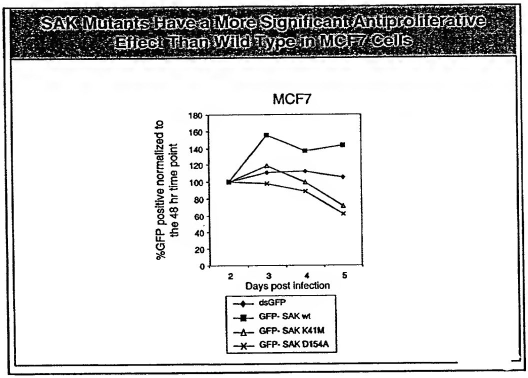

SAK Mutants Have a More Significant Antiproliferative Effect Than Wild Type in MCF7 Cells
MCF7
%GFP positive normalized to the 48 hr time point
180
160
140
120
100
80
60
40
20
0
2
3
4
5
Days post infection
dsGFP
GFP- SAK wt
GFP- SAK K41M
GFP- SAK D154A

Figure 7

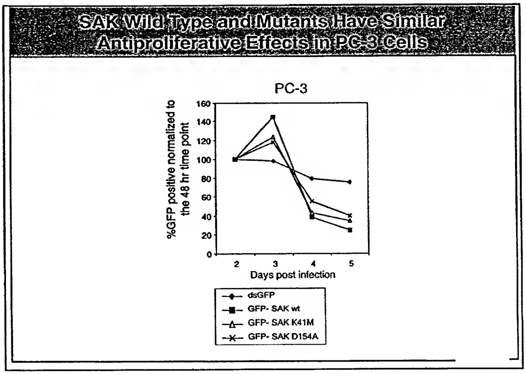

SAK Wild Type and Mutants Have Similar Antiproliferative Effects in PC-3 Cells
PC-3
%GFP positive normalized to the 48 hr time point
160
140
120
100
80
60
40
20
0
2
3
4
5
Days post infection
dsGFP
GFP- SAK wt
GFP- SAK K41M
GFP- SAK D154A

Figure 8

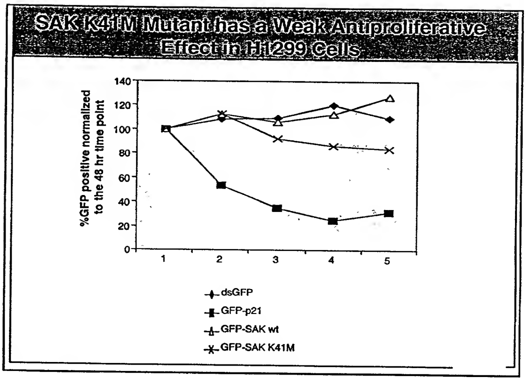

SAK K41M Mutant has a Weak Antiproliferative Effect in H1299 Cells
%GFP positive normalized to the 48 hr time point
140
120
100
80
60
40
20
0
1
2
3
4
5
dsGFP
GFP-p21
GFP-SAK wt
GFP-SAK K41M

Figure 9

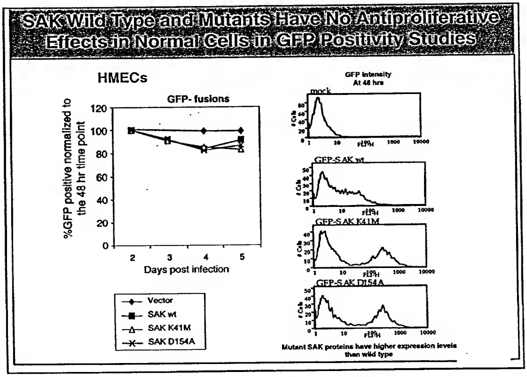

SAK Wild Type and Mutants Have No Antiproliferative Effects in Normal Cells in GFP Positivity Studies
HMECs
GFP- fusions
%GFP positive normalized to the 48 hr time point
Days post infection
Vector
SAK wt
SAK K41M
SAK D154A
GFP intensity At 48 hrs
mock
GFP-SAK wt
GFP-SAK K41M
GFP-SAK D154A
Cells
FL1-H
Mutant SAK proteins have higher expression levels than wild type

Figure 10

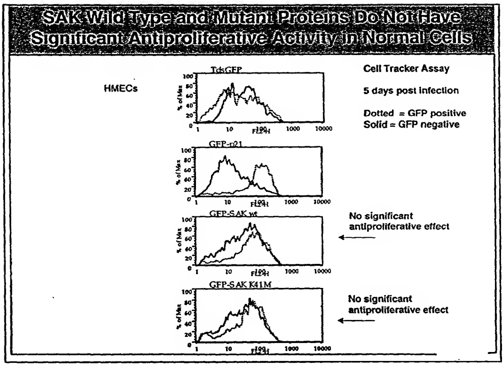

SAK Wild Type and Mutant Proteins Do Not Have Significant Antiproliferative Activity in Normal Cells
HMECs
TdsGFP
GFP-p21
GFP-SAK wt
GFP-SAK K41M
% of Max
FL2-H
Cell Tracker Assay
5 days post infection
Dotted = GFP positive
Solid = GFP negative
No significant antiproliferative effect
No significant antiproliferative effect

Figure 11

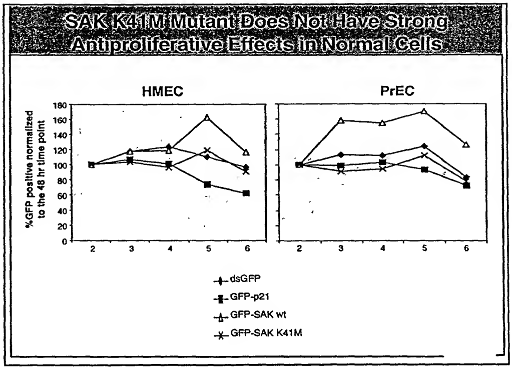
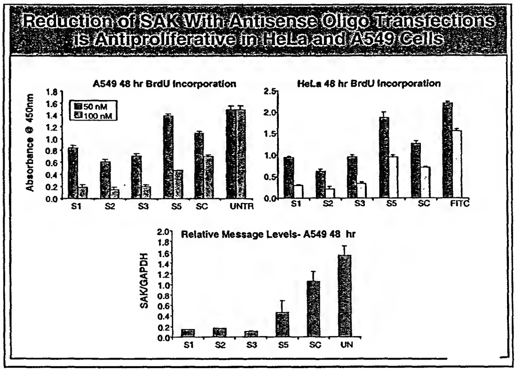

SAK K41M Mutant Does Not Have Strong Antiproliferative Effects in Normal Cells
HMEC
PrEC
%GFP positive normalized to the 48 hr time point
0
20
40
60
80
100
120
140
160
180
2
3
4
5
6
dsGFP
GFP-p21
GFP-SAK wt
GFP-SAK K41M

Figure 12



Reduction of SAK With Antisense Oligo Transfections is Antiproliferative in HeLa and A549 Cells
A549 48 hr BrdU Incorporation
Absorbance @ 450nm
50 nM
100 nM
S1
S2
S3
S5
SC
UNTR
HeLa 48 hr BrdU Incorporation
FITC
Relative Message Levels- A549 48 hr
SAK/GAPDH
UN

Figure 13

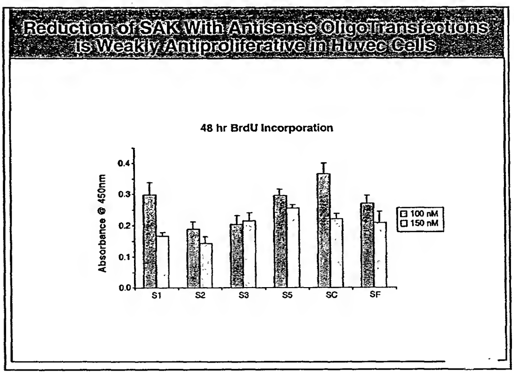

Reduction of SAK With Antisense OligoTransfections
is Weakly Antiproliferative in Huvec Cells
48 hr BrdU Incorporation
Absorbance @ 450nm
0.0
0.1
0.2
0.3
0.4
S1
S2
S3
S5
SC
SF
100 nM
150 nM

Figure 14

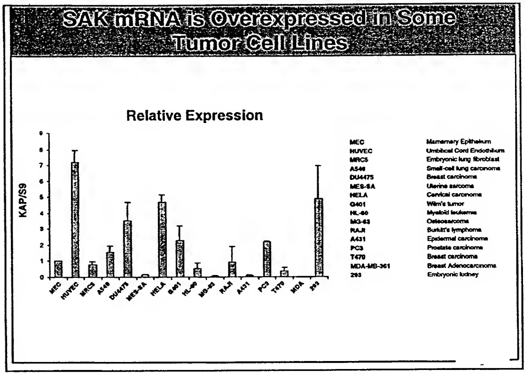

SAK mRNA is Overexpressed in Some Tumor Cell Lines
Relative Expression
KAP/S9
0
1
2
3
4
5
6
7
8
9
MEC
HUVEC
MRC5
A549
DU4475
MES-SA
HELA
G401
HL-60
MG-63
RAJI
A431
PC3
T470
MDA
293
MEC Mamemary Epithelium
HUVEC Umbilical Cord Endothilium
MRC5 Embryonic lung fibroblast
A549 Small-cell lung carcinoma
DU4475 Breast carcinoma
MES-SA Uterine sarcoma
HELA Cervical carcinoma
G401 Wilm's tumor
HL-60 Myeloid leukemia
MG-63 Osteosarcoma
RAJI Burkitt's lymphoma
A431 Epidermal carcinoma
PC3 Prostate carcinoma
T470 Breast carcinoma
MDA-MB-361 Breast Adenocarcinoma
293 Embryonic kidney

Figure 15

SAK Summary

Identification

Proteomics- Chk2 interacting protein

Functional Studies

Dominant Negative Studies

- Mutant SAK has a much stronger antiproliferative phenotype than the wild type SAK in tumor cells while neither wild type or mutant SAK is antiproliferative in normal cells.
- The higher expression level of the mutant SAK relative to wild type makes it difficult of validate SAK only by the dominant negative strategy

Antisense Studies

- Preliminary studies suggests that inhibition of SAK mRNA with antisense oligos is antiproliferative in A549 and Hela cells

Literature

- Strong supporting literature shows antisense reduction of mouse SAK is antiproliferative and that the mouse SAK knockout results in increased cell cycle arrest and apoptosis

Figure 16

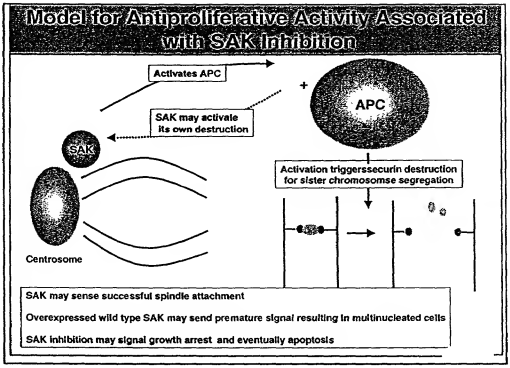
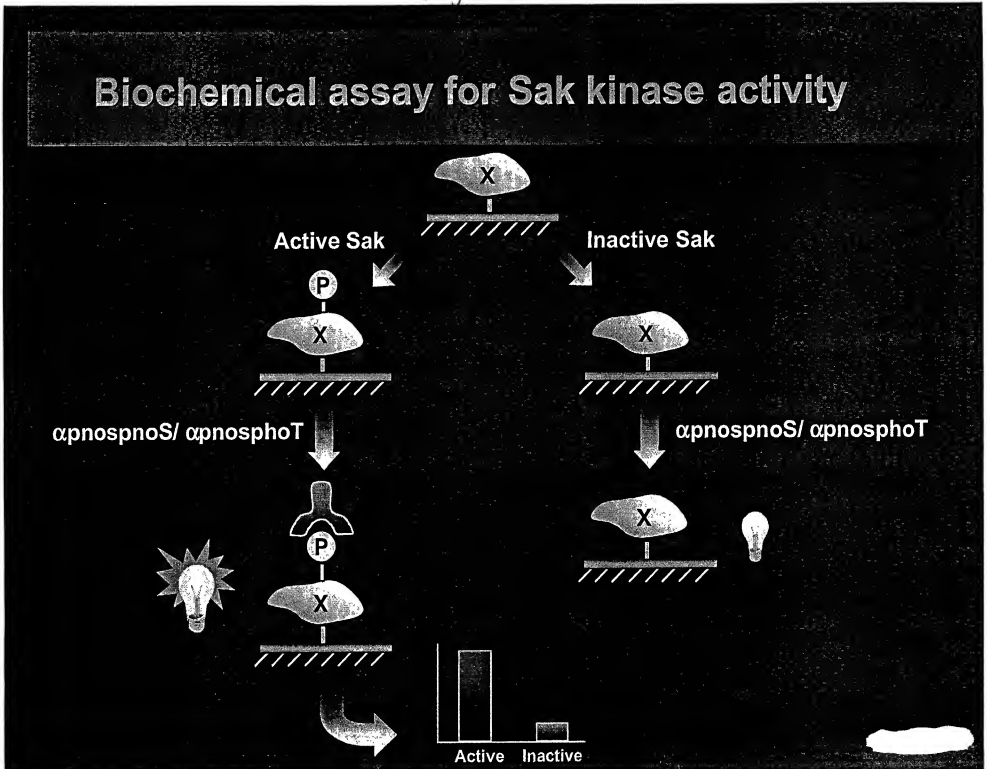

Model for Antiproliferative Activity Associated with SAK Inhibition
Activates APC
+
APC
SAK may activate its own destruction
SAK
Centrosome
Activation triggerssecurin destruction for sister chromosomse segregation
SAK may sense successful spindle attachment
Overexpressed wild type SAK may send premature signal resulting in multinucleated cells
SAK inhibition may signal growth arrest and eventually apoptosis

Figure 17



Biochemical assay for Sak kinase activity
X
Active Sak
Inactive Sak
P
X
X
αpnospnoS/ αpnosphoT
αpnospnoS/ αpnosphoT
P
X
X
Active
Inactive

Figure 18

Protocol for Sak Autophosphorylation Assay

Bind Sak from *E. coli* lysates to Ni-NTA agarose O/N at 4°C

↓

Wash Ni-NTA with lysis buffer (20 mM Hepes,pH 7.2, 0.5 M NaCl, 0.5% Tween-20, 25 mM β-glycerol phosphate, 1 mM NaF, 1 mM Na_3VO_4, 1 mM NaPyP, 10% glycerol

↓

Wash Ni-NTA with kinase buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM Na_3VO_4)

↓

Resuspend resin-bound Sak in 10 μL kinase buffer
Add 10 μL of labeling mix (20 mM $MgCl_2$, 2 mM $MnCl_2$, 0.2 mM ATP, 0.5 μCi/μL γ-^{32}P ATP in kinase buffer
Incubate at 30°C, 15 min.

Figure 19

Autophosphorylation Activity of Sak Produced in *E. coli*